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РОССИЙСКОЕ АГЕНТСТВО ПО ПАТЕНТАМ И ТОВАРНЫМ ЗНАКАМ
(РОСПАТЕНТ)
ФЕДЕРАЛЬНЫЙ ИНСТИТУТ ПРОМЫШЛЕННОЙ СОБСТВЕННОСТИ

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С П Р А В К А

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Название изобретения METHOD FOR PRODUCING L-AMINO ACID USING BACTERIA BELONGING TO THE GENUS ESCHERICHIA

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METHOD FOR PRODUCING L-AMINO ACID USING BACTERIA BELONGING TO THE GENUS ESCHERICHIA

Technical field

The present invention relates to biotechnology, specifically to a method for producing L-amino acids by fermentation and more specifically to genes derived from bacteria *Escherichia coli*. The genes are useful for improvement of L-amino acid productivity, for example, L-threonine and L-valine.

Background art

Conventionally the L-amino acids have been industrially produced by method of fermentation utilizing strains of microorganisms obtained from natural sources or mutant of the same especially modified to enhance L-amino acid productivity.

There have been disclosed many techniques to enhance L-amino acid productivity, for example, by transformation of microorganism by recombinant DNA (see, for example, US patent No. 4,278,765). These techniques based on the increasing of activities of the enzymes involved into amino acid biosynthesis and/or desensitizing the target enzymes to the feedback inhibition by produced L-amino acid (see, for example, Japanese Laid-open application No56-18596 (1981), WO 95/16042 or US patent Nos. 5,661,012 and 6,040,160).

On the other hand, increased L-amino acid excretion can enhance the productivity of strain producing L-amino acid. Strain of bacterium belonging to the genus *Corynebacterium* having increased expression of L-lysine excretion gene (*lysE* gene) is disclosed (WO 9723597A2). In addition, genes coding for the efflux proteins suitable for secretion of L-cysteine, L-cystine, N-acetylserine or thiazolidine derivatives are also disclosed (USA Patent No. 5,972,663).

At present several *Escherichia coli* genes coding for putative membrane proteins enhancing L-amino acid production are disclosed. Additional copy of *rhtB* gene makes a bacterium more resistant to L-homoserine and enhances production of L-homoserine, L-threonine, L-alanine, L-valine and L-isoleucine (European patent application EP994190A2). Additional copy of *rhtC* gene makes a bacterium more resistant to L-homoserine and L-threonine and enhances production of L-homoserine, L-threonine and L-leucine (European patent application EP1013765A1). Additional copy of *yahN*, *yeaS*, *yfiK* and *yggA* genes enhance production of L-glutamic acid, L-

lysine, L-threonine L-alanine, L-histidine, L-proline, L-arginine, L-valine and L-isoleucine (European patent application EP1016710A2). And though complete genome sequence of *Escherichia coli* strain K-12 is described (Blattner F.R., Plunkett G., Bloch C.A. et al., Science, 227, 1453-1474, 1997; ftp://ftp.genetics.wisc.edu/pub/sequence/ecolim52.seq.gz), there are many ORFs, the function of which still remain unknown.

Disclosure of the invention

An object of present invention is to enhance the productivity of L-amino acid producing strains and to provide a method for producing L-amino acid, for example, L-threonine and L-valine, using the strains.

This aim was achieved by identifying genes coding for proteins, which are not involved into biosynthetic pathway of target L-amino acid but enhance its production. An example of such protein could be a membrane protein having L-amino acid excretion activity. Based on the analysis of complete genome sequence of *Escherichia coli,* proteins with 4 or more putative transmembrane segments (TMS) were selected. As a result of diligent screening, the present inventors have identified one gene among them, that is b1242, and thoroughly studied it. The gene b1242 has been known as putative CDS which may encode functionally unknown protein (numbers 8432 to 9079 in the sequence of GenBank accession AE000222 U00096). The gene b1242 is also known as *ychE*. Also the present inventors have found that by enhancing the activity of the protein encoded by b1242 gene the productivity of L-amino acid producing strain is enhanced. Thus the present invention has been completed.

The present inventions are as follows:

1). An L-amino acid producing bacterium belonging to the genus *Escherichia,* wherein L-amino acid production by the bacterium is enhanced by enhancing an activities of proteins as defined in the following (A) or (B) in a cell of the bacterium:

(A) a protein which comprises the amino acid sequence shown in SEQ ID NO:3 in Sequence listing;

(B) a protein which comprises an amino acid sequence including deletion, substitution, insertion or addition of one or several amino acids in the amino acid sequence shown in SEQ ID NO:3 in Sequence listing, and which has an activity of making bacterium having enhanced resistance to the L-amino acids and/or its analogs;

(hereinafter, the proteins as defined in the above (A) or (B) are referred to as "proteins of the present invention")

2). The bacterium according to the above bacterium, wherein the activities of the proteins as defined in (A) or (B) are enhanced by transformation of the bacterium with a DNA coding for the proteins as defined in (A) or (B), or by alteration of expression regulation sequence of said DNA on the chromosome of the bacterium.

3). The bacterium according to the above bacterium, wherein the transformation is performed with a multicopy vector.

4). A method for producing L-amino acid, which comprises cultivating the bacterium according to the above bacterium in a culture medium and collecting from the culture medium L-amino acid to be produced and accumulated.

5) The method according to the above method, wherein L-amino acid is L-threonine.

6). The method according to the above method, wherein the bacterium has enhanced expression of threonine operon.

7). The method according to the above method, wherein L-amino acid is L-valine.

8). The method according to the above method, wherein the bacterium has enhanced expression of *ilv* operon.

The method for producing L-amino acid includes production of L-threonine using L-threonine producing bacterium wherein activities of the proteins of the present invention such as that comprising amino acid sequence shown in SEQ ID NO:3 are enhanced. Also a method for producing L-amino acid includes production of L-valine using L-valine producing bacterium wherein activities of the proteins of the present invention such as that comprising amino acid sequence shown in SEQ ID NO:3 are enhanced.

The present invention will be explained in detail below.

The bacterium of the present invention is an L-amino acid producing bacterium belonging to the genus *Escherichia,* wherein L-amino acid production by the bacterium is enhanced by enhancing an activity of the proteins of the present invention in a cell of the bacterium.

A bacterium of present invention is L-amino acid producing bacterium belonging to the genus *Escherichia* having enhanced activities of proteins, which

enhance the productivity of the target L-amino acid. Concretely the bacterium of present invention is L-amino acid producing bacterium belonging to the genus *Escherichia* which has enhanced activities of the proteins of the present invention. More concretely the bacterium of present invention harbors the DNA having overexpressed b1242 gene on chromosomal DNA or plasmid in the bacterium and has enhanced ability to produce L-amino acid, for example L-threonine and/or L-valine,

The proteins of the present invention include ones as defined in the following (A) or (B):

(A) a protein which comprises the amino acid sequence shown in SEQ ID NO:3 in Sequence listing;

(B) a protein which comprises an amino acid sequence including deletion, substitution, insertion or addition of one or several amino acids in the amino acid sequence shown in SEQ ID NO:3 in Sequence listing, and which has an activity of making bacterium having enhanced resistance to the L-amino acids and/or its analogs;

The number of "several" amino acids differs depending on the position or the type of amino acid residues in the three-dimensional structure of the protein It may be 2 to 22 , preferably 2 to 11, and more preferably 2 to 5 for the protein (A).

Resistance to L-amino acids and/or its analogs means ability for bacterium to grow on a minimal medium containing L-amino acid or its analog in concentration under which the wild type or the parental strain of the bacterium cannot grow, or ability for bacterium to grow faster on a medium containing L-amino acid or its analog than the wild type or the parental strain of the bacterium. L-amino acid analogs are exemplified by DL-o-methylserine, homoserine or the like. Above mentioned concentration of L-amino acid or its analog is generally 1100 to 9600 μg/ml, preferably 3000 to 3500 in case of DL-o-methylserine and generally 330 to 3300 μg/ml, preferably 900 to 1100 μg/ml in case of homoserine

The bacterium of the present invention also includes one wherein the activities of the proteins of the present invention are enhanced by transformation of said bacterium with DNA coding for protein as defined in (A) or (B), or by alteration of expression regulation sequence of said DNA on the chromosome of the bacterium.

The DNA, which is used for modification of the bacterium of the present invention, codes for putative membrane protein. Concretely the DNA codes for protein having 4 or more transmembrane segments. Such DNA may code for proteins

having L-amino acid excretion activity. More concretely, the DNA is represented by b1242 gene. The 1242 gene can be obtained by, for example, PCR using primers having nucleotide sequence shown in SEQ ID No: 1 and 2.

Analysis of complete genome sequence of *Escherichia coli* allowed to select the genes coding for proteins having 4 or more putative TMS. Proteins with known function and transporters described by Paulsen I.T., Sliwinski M.I., Saier M.H. (*J.Mol.Biol.*, 1998, 277, 573) and Linton K.J., Higgins C.F.(*Molecular Microbiology*, 1998, 28(1), 5) were excluded from the group to be screened. As a result of diligent screening among the rest of genes, several genes coding for putative membrane exporters were chosen. And it was found the overexpression of b1242 gene enhances the L-amino acid production by L-amino acid producing strain.

The DNA of the present invention includes a DNA coding for the protein which include deletion, substitution, insertion or addition of one or several amino acids in one or more positions on the protein (A) as long as they do not lose the activity of the protein. Although the number of "several" amino acids differs depending on the position or the type of amino acid residues in the three-dimensional structure of the protein, it may be 2 to 22, preferably 2 to 11, and more preferably 2 to 5 for the protein (A). The DNA coding for substantially the same protein as the protein defined in (A) may be obtained by, for example, modification of nucleotide sequence coding for the protein defined in (A) using site-directed mutagenesis so that one or more amino acid residue will be deleted, substituted, inserted or added. Such modified DNA can be obtained by conventional methods using treatment with reagents and conditions generating mutations. Such treatment includes treatment the DNA coding for proteins of present invention with hydroxylamin or treatment the bacterium harboring the DNA with UV irradiation or reagent such as N-methyl-N'-nitro-N-nitrosoguanidine or nitrous acid.

The DNA of the present invention include variants which can be found in the different strains and variants of bacteria belonging to the genus *Escherichia* according to natural diversity. The DNA coding for such variants can be obtained by isolating the DNA, which hybridizes with b1242 gene or part of the gene under the stringent conditions, and which codes the protein enhancing L-amino acid production. The term "stringent conditions" referred to herein is a condition under which so-called specific hybrid is formed, and non-specific hybrid is not formed. For example, the stringent conditions includes a condition under which DNAs having high homology, for

instance DNAs having homology no less than 70% to each other, are hybridized. Alternatively, the stringent conditions are exemplified by conditions which comprise ordinary condition of washing in Southern hybridization, e.g., 60°C, 1 x SSC, 0.1% SDS, preferably 0.1 x SSC, 0.1% SDS. As a probe for the DNA which codes for variants and hybridizes with b1242 gene, a partial sequence of the nucleotide sequence of SEQ ID NO: 3 can also be used. Such a probe may be prepared by PCR using oligonucleotides produced based on the nucleotide sequence of SEQ ID NO: 3 as primers, and a DNA fragment containing the nucleotide sequence of SEQ ID NO: 3 as a template. When a DNA fragment in a length of about 300 bp is used as the probe, the conditions of washing for the hybridization consist of, for example, 50°C, 2 x SSC, and 0.1% SDS.

Transformation of bacterium with DNA coding for protein means introduction of the DNA into bacterium cell for example by conventional methods to increase expression of the gene coding for the protein of present invention and to enhance the activity of the protein in the bacterial cell.

Techniques for enhancement of gene expression includes methods increasing the gene copy number. Introduction of a gene into a vector that is able to function in a bacterium belonging to the genus *Escherichia* increases copy number of the gene. For such purposes multi-copy vectors can be preferably used. The multi-copy vector is exemplified by pBR322, pMW119, pUC19, pET22b or the like.

Besides, enhancement of gene expression can be achieved by introduction of multiple copies of the gene into bacterial chromosome by, for example, method of homologous recombination or the like.

In case that expression of two or more genes is enhanced, the genes may be harbored together on the same plasmid or separately on different plasmids. It is also acceptable that one of the genes is harbored on a chromosome, and the other gene is harbored on a plasmid.

On the other hand, enhancement of gene expression can be achieved by locating the DNA of the present invention under control of a potent promoter. For example, *lac* promoter, *trp* promoter, *trc* promoter, P_L promoter of lambda phage are known as potent promoters. Using the potent promoter can be combined with multiplication of gene copies.

The bacterium of the present invention can be obtained by introduction of the aforementioned DNAs into bacterium inherently having ability to produce L-amino

acid. Alternatively, the bacterium of present invention can be obtained by imparting ability to produce L-amino acid to the bacterium already harboring the DNAs. For the parent strain which is to be enhanced in activity of the proteins of the present invention, L-threonine producing bacteria belonging to the genus *Escherichia* such as strains VL2054 (VKPM B - 8067), VNIIGenetika 472T23 (US patent No. 5,631,157), VKPM B-3996 (US patent Nos. 5,175,107 and 5,976,843), KCCM-10132 (WO009660A1), KCCM-10133 (WO009661A1) or the like can be employed. Also for the parent strain which is to be enhanced in activity of the proteins of the present invention, L-valine producing bacteria belonging to the genus *Escherichia* such as H-81 (VKPM B - 8066), NRRL B-12287 and NRRL B-12288 (US patent No. 4,391,907), VKPM B-4411 (US patent No. 5,658,766), VKPM B-7707 (European patent application EP1016710A2) or the like is employed.

The bacterium of the present invention may be further enhanced expression of one or more genes which is involved in L-amino acid biosynthesis. Such gene is exemplified by threonine operon, which preferably comprises a gene encoding aspartate kinase – homoserine dehydrogenase of which feedback inhibition by L-threonine is desensitized, for L-threonine producing bacteria (Japanse Patent Publicaion No. 1-29559), and *ilvGMEDA* operon, which does not preferably express threonine deaminase and of which attenuation is suppressed, for L-valine producing bacteria (Japanese Patent Laid-Open Publication No. 8-47397).

The method of the present invention includes method for producing L-threonine, comprising steps of cultivating the bacterium of the present invention in a culture medium, to allow L-threonine to be produced and accumulated in the culture medium, and collecting L-threonine from the culture medium. Also the method of present invention includes method for producing L-valine, comprising steps of cultivating the bacterium of the present invention in a culture medium, to allow L-valine to be produced and accumulated in the culture medium, and collecting L-valine from the culture medium.

In the present invention, the cultivation, the collection and purification of L-amino acid from the medium and the like may be performed in a manner similar to the conventional fermentation method wherein an amino acid is produced using a microorganism. A medium used for culture may be either a synthetic medium or a natural medium, so long as the medium includes a carbon source and a nitrogen source and minerals and, if necessary, appropriate amounts of nutrients which the

microorganism requires for growth. The carbon source may include various carbohydrates such as glucose and sucrose, and various organic acids. Depending on the mode of assimilation of the used microorganism, alcohol including ethanol and glycerol may be used. As the nitrogen source, various ammonium salts such as ammonia and ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone, soybean-hydrolysate and digested fermentative microorganism are used. As minerals, potassium monophosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium chloride, and the like are used.

The cultivation is performed preferably under aerobic conditions such as a shaking culture, and stirring culture with aeration, at a temperature of 20 to 40 °C, preferably 30 to 38 °C. The pH of the culture is usually between 5 and 9, preferably between 6.5 and 7.2. The pH of the culture can be adjusted with ammonia, calcium carbonate, various acids, various bases, and buffers. Usually, a 1 to 5-day cultivation leads to the accumulation of the target L-amino acid in the liquid medium.

After cultivation, solids such as cells can be removed from the liquid medium by centrifugation or membrane filtration, and then the target L-amino acid can be collected and purified by ion-exchange, concentration and crystallization methods.

Brief description of the drawing

Figure 1 shows the construction of plasmid pΔlacZ.

Best Mode for Carrying out the Invention

The present invention will be more concretely explained below with reference to Examples. In the Examples an amino acid is of L-configuration unless otherwise noted.

Example 1: Cloning of the b1242 gene on the plasmid pΔlacZ.

For cloning of the b1242 gene vector pΔlacZ was used. Vector pΔlacZ is a derivative of the vector pET-22b(+) (Novagen, Madison, WI, USA). pET-22b(+) was treated by *Bgl*II and *Xba*I and ligated with polymerase chain reaction (PCR) fragment of plasmid pMB9-*lac* (Fuller F., *Gene*, 19, 43-54, 1982)treated with the same restrictases and carried P_{lac} UV5 promoter. For amplifying the P_{lac} UV5 promoter

fragment by PCR primers having sequence depicted in SEQ ID Nos: 5 and 6 were used. The resulted plasmid was supplemented with structural part of *lacZ* gene (237 bp without promoter) by cloning *Sal*I-*Bam*HI fragment of the plasmid pJEL250 (Dymakova E. *et al.*, *Genetika* (rus), *35*, 2, 181-186, 1999). Scheme for obtaining vector pΔlacZ is shown in Figure 1.

The initial material for cloning of *E.coli* b1242 putative reading frame (b1242 gene) was the PCR fragment, which was obtained using DNA from *E. coli* strain TG1 as a template. For synthesis of this fragment two primers having sequence depicted in SEQ ID Nos: 1 and 2 were used. PCR was carried out on "Perkin Elmer GeneAmp PCR System 2400" under the following conditions: 40 sec. at 95 °C, 40 sec. at 47 °C, 40 sec. at 72 °C, 30 cycles. Thus, the 735 bp linear DNA fragment contained b1242 gene was obtained. This PCR fragment was treated by *Xba*I and *Bam*HI restrictases and inserted into multicopy vector pΔlacZ previously treated by the same restrictases.

Resulted plasmid with the PCR fragment was named pYCHE and carried b1242 gene under the control of the lactose promoter (P_{lac} UV5).

Example 2: The influence of the amplified b1242 gene on resistance of *E. coli* strain TG1 to amino acids and its analogs.

E. coli strain TG1(pYCHE) and TG1 strain having a vector without insertion (control strain) were grown overnight on LB medium supplemented with ampicilline (100 μg/ml). The night cultures of all strains were diluted at 25 times in fresh LB medium supplemented with ampicilline (100 μg/ml) and IPTG (0.5 mM) and were incubated 2 hours at 37 °C with aeration. The log phase cultures were diluted in 0,9% solution of NaCl and about 1000 cells were seeded on plates with solid Adams medium supplemented with ampicilline (100 μg/ml), IPTG (0.5 mM) and amino acid or its analog. After 2 – 4 days incubation at 37 °C the differences in colony size or colony number between the TG1 strain with hybrid plasmid and control TG1 strain were registered. The results of experiments are presented in Table 1.

Table 1.

Inhibitors	Concentration in media, μg/ml	Effect on the growth of TG1 strain having plasmid pYCHE
Proline	30000	no
3,4-Dihydroproline	23	no
Isoleucine	18000	no

DL-Thiaisoleucine	1	no
o-Methylthreonine	6	no
L-Serine	2800	no
DL-Serine	3600	no
DL-Serine hydroxamate	140	no
DL-o-Methylserine	3200	R
4-Azaleucine	45	no
6-Diazo-5-oxo-L-norleucine	15	no
Valine	7	no
Methionine	38000	no
Norleucine	500	no
Cysteine	1600	no
Homoserine	1000	R
DL-β-Hydroxy-norvaline	80	no
L-Aspartic acid β-hydroxamate	100	no
Arginine	4300	no
Lysine	5000	no
S-(2-Aminoethyl)cysteine	0.75	no
Histidine	3000	no
L-Histidine hydroxamate	200	no
DL-1,2,4-Triazole-3-alanine	80	no
Phenylalanine	13000	no
p-Fluorophenylalanine	6	no
L-o-Fluorophenylalanine	1.7	no
DL-o-Fluorophenylalanine	6	no
Tryptophan	12500	no
DL-4-Fluorotryptophan	0.1	no
4-Methyltryptophan	0.25	no
7-Methyltryptophan	100	no
DL-a-Methyltrypptophan	400	no
m-Fluoro-DL-tyrosine	0.5	no

no - no differences compare to the control strain
R - more colonies or colony size

Example 3: Production of threonine by a strain having plasmid pYCHE.

The threonine producing strain VL2054 was transformed by the plasmid pYCHE carried the b1242 gene under the control of P_{lac} UV5 promoter. Obtained strain was named VL2054(pYCHE). The strain VL2054 is derivative of the strain VKPM B-3996 and carried on its chromosome:

a) the integrated threonine operon under the control of P_R promoter

b) wild type *rhtA* gene

c) the inactivated chromosomal gene encoding transhydrogenase (*tdh* gene) and inactivated kanamycin resistant gene (*kan*) gene in the Tn5 (tdh::Tn5, Kan^S)

d) mutation $ilvA_{442}$.

The strain VL2054 has been deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (Russia 113545 Moscow 1 Dorozhny proezd, 1) on January 30, 2001 under accession number VKPM B-8067.

The 5 colonies of each strain VL2054, strain VL2054(pΔlacZ) as a control strain contained plasmid without insertion and VL2054(pYCHE) were suspended in 2 ml of minimal medium ($(NH_4)_2SO_4$ – 11 g/l; NaCl – 0.4 g/l; $MgSO_4$ – 0.4 g/l; K_2HPO_4 - 1 g/l; $FeSO_4$ - 10 mg/l; $MnSO_4$ - 10 mg/l; thiamin – 0.1 mg/l; yeast extract – 0.5 g/l; glucose - 40 g/l; ampicilline - 300 mg/l if necessary) in 20-ml test tubes and were incubated overnight with aeration at 32 °C. The 0.2 ml of each night culture was transferred to the three 20-ml test tubes with 2 ml of fresh medium for fermentation with or without IPTG and cultivated at 32 °C for 45 hours with rotary shaker.

Fermentation medium composition:

$(NH_4)_2SO_4$	22 g/l
NaCl	0.8 g/l
$MgSO_4$	0.8 g/l
K_2HPO_4	2 g/l
$FeSO_4$	20 mg/l
$MnSO_4$	20 mg/l
Thiamin	0.2 mg/l
Yeast extract	1 g/l
$CaCO_3$	30 g/l
Glucose	80 g/l
Ampicilline	300 mg/l, if necessary
IPTG	0.5 mM, if necessary

After cultivation the plasmid stability and optical absorbance of the medium at 540 nm were determined by conventional methods. Accumulated amount of threonine in the medium was determined by thin layer chromatography (TLC). Liquid phase composition for TLC was as follows: isopropanol - 50 ml, acetone - 50 ml, NH_4OH (30 %) - 12 ml, H_2O - 8 ml. The results are shown in Table 2. As it is seen, the hybrid plasmid pYCHE improved the threonine accumulation by the threonine producing strain VL2054.

Table 2.

VL2054 with plasmid	IPTG	OD_{540}	Thr, g/l	Thr/OD
no	-	21	4.8	0.23
	+	20	4.7	0.24
pΔlacZ	-	16	4.6	0.29
	+	13	3.0	0.23
pYCHE	-	20	6.2	0.31
	+	20	7.0	0.35

Example 4: Production of valine by a strain having plasmids pYGAZH.

The valine producing strain H-81 was transformed by the plasmid pYCHE carried the b1242 gene under the control of P_{lac} UV5 promoter. The strain H-81 has been deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (Russia 113545 Moscow 1 Dorozhny proezd, 1) on January 30, 2001 under accession number VKPM B - 8066.

The 5 colonies of each strain H-81, H-81(pΔlacZ) as a control strain contained plasmid without insertion and H-81(pYCHE) were suspended in 2 ml of minimal medium ($(NH_4)_2SO_4$ - 18 g/l, K_2HPO_4 - 1.8 g/l, $MgSO_4$ - 1.2 g/l, thiamin - 0.1 mg/l, yeast extract – 0.5 g/l, glucose - 60 g/l, ampicilline - 300 mg/l, if necessary) in 20-ml test tubes and were incubated overnight with aeration at 32 °C. The 0.2 ml of each night culture was transferred to the three 20-ml test tubes with 2 ml of fresh medium for fermentation with or without IPTG and cultivated at 32 °C for 45 hours with rotary shaker.

Fermentation medium composition:

$(NH_4)_2SO_4$	18 g/l,
K_2HPO_4	1.8 g/l,
$MgSO_4$	1.2 g/l,
$CaCO_3$	20 g/l,
Thiamin	0.1 mg/l,
Glucose	60 g/l,
Ampicilline	300 mg/l, if necessary
IPTG	0.5 mM, if necessary

After cultivation the plasmid stability and optical absorbance of the medium at 540 nm were determined by conventional methods. Accumulated amount of valine in the medium was determined by TLC. Liquid phase composition for TLC was as

follows: isopropanol - 80 ml, ethylacetate - 80 ml, NH_4OH (30 %) - 15 ml, H_2O - 45 ml. The results are shown in Table 3. As it is seen, the hybrid plasmid pYCHE improved the valine accumulation by the valine producing strain H-81.

Table 3

H-81 with plasmid	IPTG	OD_{540}	Val, g/l	Val/OD
no	-	34	11.6	0.34
	+	34	11.7	0.34
pΔlacZ	-	34	10.5	0.31
	+	20	7.8	0.39
pYCHE	-	32	14.0	0.44
	+	30	13.9	0.46

SEQUENCE LISTING

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Gly Lys Leu Gly Glu Asp Lys Gln Asn Lys Gln Glu Lys Ser Glu Thr
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gcg gta cgt gaa agc att ggt gtg gtg cca ctg gcg ttg ccg ttg atg   384
Ala Val Arg Glu Ser Ile Gly Val Val Pro Leu Ala Leu Pro Leu Met
        115                 120                 125
gcg ggg cca ggg gcg atc agt tct acc atc gtc tgg ggt acg cgt tat   432
Ala Gly Pro Gly Ala Ile Ser Ser Thr Ile Val Trp Gly Thr Arg Tyr
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cac agc att agc tat ctg ttt ggt ttc ttt gtg gct att gca ttg ttc   480
His Ser Ile Ser Tyr Leu Phe Gly Phe Phe Val Ala Ile Ala Leu Phe
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gct tta tgt tgt tgg gga ttg ttc cgc atg gca ccg tgg ctg gta cgg   528
Ala Leu Cys Cys Trp Gly Leu Phe Arg Met Ala Pro Trp Leu Val Arg
                165                 170                 175
gtt tta cgc cag acc ggc atc aac gtg att acg cgt att atg ggg cta   576
Val Leu Arg Gln Thr Gly Ile Asn Val Ile Thr Arg Ile Met Gly Leu
            180                 185                 190
ttg ctg atg gca ttg ggg att gaa ttt atc gtt act ggt att aag ggg   624
Leu Leu Met Ala Leu Gly Ile Glu Phe Ile Val Thr Gly Ile Lys Gly
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Ser Met Thr Ser Tyr Gln Thr Ala Ala Ala Arg Asn Lys Thr Asn Leu
        35                  40                  45
Thr Ala Asn Leu Ser Val Ala Ile Ile Leu Trp Ile Ser Leu Phe Leu
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Gly Asp Thr Ile Leu Gln Leu Phe Gly Ile Ser Ile Asp Ser Phe Arg
 65                  70                  75                  80
Ile Ala Gly Gly Ile Leu Val Val Thr Ile Ala Met Ser Met Ile Ser
                85                  90                  95
Gly Lys Leu Gly Glu Asp Lys Gln Asn Lys Gln Glu Lys Ser Glu Thr
            100                 105                 110
Ala Val Arg Glu Ser Ile Gly Val Val Pro Leu Ala Leu Pro Leu Met
        115                 120                 125
Ala Gly Pro Gly Ala Ile Ser Ser Thr Ile Val Trp Gly Thr Arg Tyr
    130                 135                 140
His Ser Ile Ser Tyr Leu Phe Gly Phe Phe Val Ala Ile Ala Leu Phe
145                 150                 155                 160
Ala Leu Cys Cys Trp Gly Leu Phe Arg Met Ala Pro Trp Leu Val Arg
                165                 170                 175
Val Leu Arg Gln Thr Gly Ile Asn Val Ile Thr Arg Ile Met Gly Leu
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What is claimed is:

1. An L-amino acid producing bacterium belonging to the genus *Escherichia* wherein L-amino acid production by said bacterium is enhanced by enhancing activities of proteins as defined in the following (A) or (B) in a cell of said bacterium:

(A) a protein which comprises the amino acid sequence shown in SEQ ID NO:3 in Sequence listing;

(B) a protein which comprises an amino acid sequence including deletion, substitution, insertion or addition of one or several amino acids in the amino acid sequence shown in SEQ ID NO:3 in Sequence listing, and which has an activity of making bacterium having enhanced resistance to L-amino acids and/or its analogs;

2. The bacterium according to the claim 1, wherein said activities of proteins as defined as (A) or (B) are enhanced by transformation of said bacterium with DNA coding for protein as defined in (A) or (B), or by alteration of expression regulation sequence of said DNA on the chromosome of the bacterium.

3. The bacterium according to the claim 2, wherein the transformation is performed with a multicopy vector.

4. A method for producing L-amino acid, which comprises cultivating the bacterium according to any of claims 1 to 3 in a culture medium and collecting from the culture medium L-amino acid to be produced and accumulated.

5. The method according to claim 4, wherein L-amino acid is L-threonine.

6. The method according to claims 5, wherein the bacterium has enhanced expression of threonine operon.

7. The method according to claim 4, wherein L-amino acid is L-valine.

8. The method according to claims 7, wherein the bacterium has enhanced expression of *ilv* operon.

Abstract of disclosure

There is provided a method for producing L-threonine and L-valine using bacterium belonging to the genus *Escherichia* wherein L-amino acid productivity of said bacterium is enhanced by enhancing an activity of proteins coded by b1242 gene.

Figure 1. Scheme for construction plasmid pΔlacZ.